On Farm

Research

On Farm Series | Cotton Research & Development Corporation

FINAL REPORT 2006

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

CRDC Project Number: CRC69 **OR Cotton CRC Project Number:** 1.01.02

Project Title: Rhizospehere biological functions as influenced by GM cotton

Project Commencement Date: 01/07/2002 **Project Completion Date:** 02/06/2006

CRDC Program: On Farm **OR CRC Program:** The Farm

Part 2 – Contact Details

Administrator: Linda Leavitt
Organisation: CSIRO Entomology
Postal Address: GPO Box 1700, CANBERRA, ACT 2601
Ph: 02 6246 4030 **Fax:** 02 6246 4094 **E-mail:** linda.leavitt@csiro.au

Principal Researcher: Oliver Knox
Organisation: CSIRO Entomology
Postal Address: 21888 Kamilaroi Highway, Narrabri, NSW 2390
Ph: 0267991583 **Fax:** 0267931186 **E-mail:** oliver.knox@csiro.au

Supervisor: Gupta Vadakattu
Organisation: CSIRO Entomology
Postal Address: Gate 5, Waite Road, Urrbrae, Adelaide, SA 5064
Ph: 0883038579 **Fax:** 0883038465/8550 **E-mail:** gupta.vadakattu.csiro.au

Signature of Research Provider Representative: ______________________________

Part 3 – Final Report Guide (due 31 October 2006)

Background

The soil microbiota and biological functions play a critical role in the sustainability of cotton productivity along with the maintenance and improvement of environmental health. In a workshop, held at Narrabri during December 2001, research on soil microbiota and biological health was identified as a priority.

The well-justified caution that has been shown in Australia, with regards to the introduction of Bt cotton and the development of insect resistance, should also be applied to the preservation of soil sustainability to protect the soil's biological function. Rhizosphere (the zone of soil bordering and influenced by plant roots) contains a large majority of the soil's biota populations (>10-fold of that in the bulk soil) and the plant-microbe interaction in the rhizosphere is one of the major factors regulating the health and growth of the plant. In Australian soils, the rhizosphere harbours and accounts for >50% of total soil microbial population in terms of both number and activity. It is also widely acknowledged that rhizodeposition, including root exudates, govern which organisms reside in the rhizosphere (Lynch, 1994). Therefore any change to the quality of rhizosphere exudate will potentially modify the dynamics of the soil biota composition and activity and may cause changes to both deleterious and beneficial microflora.

Genetically modified (GM) plants, through (1) the products of introduced genes, and (2) unintentionally modified rhizosphere chemistry, have the potential to significantly change these microbial dynamics, plant-microbe interaction, and essential ecosystem functions such as nutrient mineralisation, disease incidence, carbon turnover and plant growth. Little data is available on the consequences of plant-microbe-soil interactions due to sustained Bt expression and/or presence of Bt protein in the rhizosphere. Preliminary evidence from Drs Gupta, Watson and Roberts indicated that: (1) Bt-cotton plants (single gene, Cry1Ac) produce Bt protein in the roots, (2) Bt-cotton plants exude Bt protein from roots, (3) microbial populations associated with Bt-cotton (single gene) and conventional cotton are significantly different, and (4) Roundup-Ready cotton had significantly different microbial activity associated with its decomposing tissue compared to the parental non-GM cotton variety (Gupta *et al*. 2001). With the introduction of 2-gene Bt and Roundup Ready/Bt cotton, research was required to attempt to understand their potential impact on the rhizosphere microflora and the sustainability of soil microbial function.

References:

- Gupta, V.V.S.R., Roberts, G.N., Neate, S.M., Crisp, .P, McClure, .S and Watson, S.K. (2001) "Impact of Bt-cotton on biological processes in Australian soils" Proceedings from the 4th Pacific Rim Conference on the Biotechnology of *Bacillus thuringiensis* and its Environmental Impact. Canberra, Australia. pp. 87.
- Lynch, J.M. (1994). The rhizosphere – form and function. Applied Soil Ecology, 1:193-198.

Objectives

1. List the project objectives and the extent to which these have been achieved.

- Establish a position at Narrabri and develop a suite of microbial techniques within the ACRI for investigation of the rhizosphere microbiota under GM and non-GM cotton.

Dr Oliver Knox was appointed to the ACRI, Narrabri as of the 2nd of June 2003. The project was up and running with glasshouse experiments within the month. A significant progress in establishing a working research capacity was achieved within the first year. Briefly, (i) a rhizosphere sampling strategy had been developed and refined, (ii) field experiments had been planted and monitored, (iii) measurements of bacterial and fungal populations from rhizosphere soils were being undertaken, (iv)

facilities to conduct soil respiration microbial biomass measurements at Narrabri were established, and (v) DNA was being extracted from soils for further analysis in Adelaide.

- To determine if there are significant differences in the total rhizosphere microfloral populations (diversity and activity) between conventional (parental) cotton and the new GM-cotton varieties (2 gene and RRI). Comparisons between selected conventional cotton varieties will also be made.

Total rhizosphere population monitoring through traditional culturable techniques were made over three seasons in four field experiments. These were conducted at the ACRI on Sicot 189 and its transgenic offspring; 189RR, 289i, 289B and 289BRR. No reproducible or continually significant differences in results were observed in field experiments, although glasshouse experiments indicated consistent differences. For example, molecular techniques, which assess populations based on the DNA they contained, indicated clear cultivar differences in the glasshouse. These differences were either reduced or completely obscured when rhizosphere soil from field grown plants was investigated. A GM versus non-GM difference was discernable from the molecular results as the assessed traits did not branch together when dendograms of population similarity were compared. Respiration measurements were made, as an indicator of microbial activity, and also showed no differences under GM and non-GM varieties. In the final year, to reflect the changing preference of seed orders, Sicot 71 and its transgenic family was included. In most assessments there remained no difference in measured microbial parameters, however, some results indicated a cotton ‘family’ difference between Sicot 189 and its transgenic offspring when compared to Sicot 71 and its transgenic counterpart.

- To assess which of the microfloral populations associated with major soil biological functions (e.g. Nitrifying microorganisms, P solubilisers, or root growth) are impacted upon in these rhizosphere soils.

As previously mentioned, respiration was assessed as an overall measure of microbial activity and was not observed to differ between the rhizosphere soils collected from under the same cultivar family. Populations of ammonium oxidiser (AO) bacteria were also assessed. The conversion of ammonium to nitrate in soil is carried out by AO (also known as nitrifying bacteria) and so the role of these bacteria in the soil is very important for plant N uptake. Plants more readily take up N in the form of nitrate (NO_3) than they do other nitrogenous chemicals, such as ammonium (NH_4^+), which is produced by nitrogen fixation, decomposing organic matter and is the basis of most fertilizer applications. Differences were observed in AO populations between 289BR and the other members of the Sicot 189 family, however, these differences were not maintained over the season. In the first year of the project some particular enzyme functions were also assessed, but no differences between GM and non-GM cultivars were observed. Cotton obtains much of its phosphorous (P) through establishment of symbiosis with mycorrhizal fungi. An investigation undertaken in collaboration with Dr D. Nehl (NSW DPI) indicated that GM and non-GM cotton are colonized to the same extent by mycorrhizal fungi. Given that the assessed crops did not show any signs of P deficiency it was assumed that this relationship was still working and that there was no impact of GM technology on cotton’s ability to obtain P. Previous experiments with single Bt gene cotton varieties in pot culture experiments in Adelaide also did not find significant differences in mycorrhizal colonization levels compared to their conventional counterparts.

- To determine the relationship, if any, between the changes in microfloral and faunal functions with growth and health of the new GM cotton plants.

As no changes in the microflora were observed in response to the GM traits with any consistency it was not possible to identify individual microbes or functional groups that could be analysed further. In the evaluated field trials a yield analysis was always conducted and, under conventional management of the crop, no loss in yield was observed with the use of GM technology. Some rhizosphere soil functional analysis was undertaken in preliminary trials within the rotation work of

Nilantha Hulugalle and Ian Rochester. This preliminary work indicated that rotation can have significant effects on the ability of the associated microbial community to carry out a number of functions and also influence their responsiveness to changes in the system. Information on the dynamics of microbial communities important for sustainable delivery of beneficial functions as influenced by management practices such as rotation and stubble management is not available.

Methods

2. Detail the methodology and justify the methodology used. Include any discoveries in methods that may benefit other related research.

Rhizosphere recovery:

Over the first year of the project and during both the glasshouse and field evaluation work we set out to establish a sampling protocol that ensured recovery and analysis of cotton rhizosphere soil. Rhizosphere soil is the soil that borders the plant root and is directly influenced by it. Because of the influence that the root has on this soil, the extent of the rhizosphere can vary greatly depending on a number of factors. These include the soil type, the root type (monocot versus dicot - fibrous versus tap and lateral), the exudate causing influence (volatile gas or dissolved potential food source), and the indicator being assessed. Because we were working with cotton, its tap and lateral root system, in a heavy clay soil, and were looking for differences in microbial populations we had to be sure the soil we sampled as rhizosphere was capable of reflecting this.

Experiments were established in which cores (2 cm diameter by 15 cm deep) were taken adjacent to and at 4 cm intervals from the tap roots point of entry into the soil. The bottom 2 cm of these cores were analysed for total recoverable microbial populations on a number of selective media (PSA, TSA and Copiotroph). Results showed no differences between the cores over distance from the tap root. A second assessment was made. This time a core was taken adjacent to the root, but for comparison the root system was removed from the soil, with the aid of a fork, and soil attached to the root recovered. Microbial populations from soil attached to the root surface were between 6 and 150 times higher than those recovered from the core. Sampling for cotton rhizosphere soil was, for the purposes of this project and others, defined as being the soil found in direct contact with the root surface and sampling techniques were modified to ensure collection of this soil.

Bacterial:

Bacterial recovery using standard culturable techniques suffers from two main drawbacks. The first of these is that only a small proportion (~1-10%) of the total soil microbial population will actually grow on agar under laboratory conditions. The second is that the technique is prone to large variation. The time consuming and labour intensive process of this plate based technique make it difficult to reduce the variation sufficiently through increasing the number of assayed replicates. Despite these drawbacks use of a number of selective and general agars provides the most suitable method to capture potential variation in the culturable microbial populations under GM and non-GM cotton.

Bacterial recovery was carried out from ~1g of rhizosphere soil, extracted in 10 mL of phosphate buffer (8 g NaCl, 0.17 g KH_2PO_4, 0.6 g K_2HPO_4, adjusted to pH 6.8 in 1 L of water), with shaking at 180 revolutions per minute for 1 hour. A stepped dilution series was prepared by adding 100 µL of the extract to 900 µL of phosphate buffer, then repeating this using the previous dilution as the next source of the 100 µL. Six dilution steps were made in this way. Three replicate 10 µL drops of each dilution were placed onto marked agar plates (two plates divided into quarters with each quarter labelled according to a particular dilution), allowed to air dry onto the agar, and incubated at 23°C for 24 to 48 hours. After this time dilutions that had produced around 10 to 100 colony forming units per 10 µL drop were counted and the actual bacterial population estimated from this.

Agars used for the bacterial recovery included Pseudomonad Selective Agar (PSA), 1/10th strength Tryptone Soy Agar (TSA) and Copiotroph agar (CA – 0.5 g $MgSO_4.7H_2O$, 0.5 g KNO_3, 1.3 g $K_2HPO_4.3H2O$, 0.06 g $Ca(NO_3)_2.4H2O$, 2.5 g Glucose, 0.6 g Casein Acid Hydrolysates, 10 g Bacteriological agar, pH 6.8-7 in 1 L sterile distilled water). All agar media were sterilised at 115°C for 15 minutes and allowed to cool prior to either the addition of antibiotics or pouring. The antibiotic cycloheximide was added to all these media at a final concentration of 10 µg/mL to inhibit fungal

growth. In some experiments when the potential for the *npt*II gene, introduced as a marker in the transgenic construction, was being considered as a possible source of difference between GM and non-GM cultivars, kanamycin was included with the agar at 25 µg/mL final concentration.

Fungal:

Fungal recovery was carried out from ~1g of rhizosphere soil, extracted with shaking (at 180 revolutions per minute) for 1 hour in 10 mL of phosphate buffer (8 g NaCl, 0.17 g KH_2PO_4, 0.6 g K_2HPO_4, adjusted to pH 6.8 in 1 L of water). A dilution series was prepared in further amounts of phosphate buffer, a 100 µL aliquot of each dilution was added to Rose Bengal and Czapek-Dox agar plates and spread with the use of a sterile glass spreader (a.k.a. hockey stick). Established plates were sealed with Parafilm® and incubated at 23°C for 5 days to allow colony development.

The media choices of Rose Bengal (RB) and Czapek-Dox (CD) agar were made due to the slight differences in the types of fungi they recover. RB is suitable for the growth of most fungi and yeast, but the presence of the Rose Bengal inhibits fast growth, allowing slower growing fungi to be detected. RB also causes yeast and some fungal cultures to develop a pink colouring that can be utilised in identification. CD contains sodium nitrate as the sole source of nitrogen, so only fungi capable of obtaining their nitrogen from sodium nitrate grow. The selective pressure that the sodium nitrate causes was made evident over the period of this project in so far as CD agar plates had significantly lower numbers of fungal colony forming units, when compared to its RB counterparts, from almost every sample.

Respiration:

Respiration has been measured in one of two ways throughout the project. These have been either assessing the rate of CO_2 evolution from a known mass of rhizosphere soil within the laboratory or by taking in field measurements of the soil (microbial and plant) respiration as a function of area. In both methods evolved CO_2 is trapped with a sodium hydroxide solution and CO_2 content analysed using dual endpoint titration with HCl. The method is prone to variation and has not revealed any significant differences between GM and conventional cultivars, regardless as to whether the assessment was on lab or field based studies.

At on sampling in 2005/06 a respiration difference were observed between the Sicot 71 and Sicot 189 families. Reasons behind this observation are unknown and subsequent and previous data sets did not allow interrogation of the system in this format, however, the shorter season and plant size of the 71 family, compared to 189, might have below ground implications for root development, timing of root senescence, and could provide a tool for reduced greenhouse gas emissions. Further investigation of this is required, as is a more detailed interrogation of the rooting properties of the various families of available cultivars. Investigation of the latter of these has been proposed by Dr James Neilsen, in collaboration with Dr Warwick Stiller under a new CRDC funded project.

Biomass:

Microbial biomass was assessed using a method that measures the ninhydrin reactive nitrogen within the soil. Amino-acids, peptides and proteinaceous molecules represent a labile pool of the soil organic matter that is largely stored within the microbial population or readily utilised by it upon release. Ninhydrin complexes with amino-acids, peptides and proteins to form a purple compound, which can be measured and quantified. The methodology works by assessing the ninhydrin reactive N of soils prior and post fumigation with chloroform and assessing the difference. The chloroform fumigation kills the microbiota liberating their amino-acids, peptides and proteins and the generated values can be utilised to infer microbial biomass from within soil samples.

The development and implementation of this technique at the ACRI has facilitated its use in other research projects. The CRC summer scholarship of Nicholas Luelf utilised this to demonstrate differences in some of the rotation work undertaken by Dr Nilantha Hulugalle. More recently, interest in microbial biomass measurements, again under different rotations, has been expressed by Dr Ian Rochester having obtained some preliminary information from the CSIRO Entomology soils microbiology group.

Ammonium Oxidisers:

The ammonium oxidiser (AO) populations were analysed from a suspension of ~1g of rhizosphere soil in 10 mL of phosphate buffer. This suspension was used to prepare a dilution series, in two fold steps, over 10 wells of a 96 well plates, thus facilitating the analysis of 8 replicates of each sample per plate. The plates are established with a minimal media containing ammonium and are sealed and incubated in the dark, at 21°C for 21 days. After this time Griess reagent is used to indicate the presence of nitrite, the last pink wells in the dilution series are scored and the most probable number (MPN) of AO bacteria calculated.

DGGE:

DNA was extracted from 0.5g of soil using a salt and bead beater method based around that published by Griffith *et al.* (2000. Applied and Environmental Microbiology 66:5488-5491) and adapted for high throughput of heavy clay soils by Dr Alice Simpson (University of Sydney). DNA extractions were sent to Adelaide for Polymerase chain reaction (PCR) of general and specific bacterial 16SrRNA genes, population profiling and analysis with denaturant gradient gel electrophoresis (DGGE).

The PCR reactions and subsequent DGGE population fingerprints were generated using different primer sets. These were 16S rDNA bacteria specific primers (27f and 534r) and *Burkholderia* specific primers (Burk3 and BurkR nested within Burk3 and R1378) for *Burkholderia* community structure.

Denaturant gradient gel electrophoresis was performed on the CBS Scientific DGGE-2400 machine at 135V for 19 hours. Sample separation occurred best on gels consisting of 8% acrylamide/bis, with bacteria having a 35-65% and *Burkholderia* a 40-70% denaturant gradient. This generated a bacteria fingerprint consisting of 49 identified band types, with each sample having 17-36 individual bands. For *Burkholderia*, the number of band types was 34, with each sample consisting of 4-15 bands.

Yield:

Yield was monitored in every established and monitored field trial over the course of the research project. This was achieved using the breeders ‘red picker’ with its suspended weighing platform, with data collected in either one or two passes through the field providing between 16 to 40 individual picked weights per field experiment. Data from each variety plot was treated as an individual measure of cultivar performance, adjusted for gin turnout and converted to bales per acre and bale per hectare estimates. These estimates were analysed using ANOVA for differences in cultivar performance.

Quantitative ELISA:

Prior to this project some evidence had been obtained to suggest that Cry1Ac was being exuded from the roots of transgenic Ingard® cotton. With the two gene, Cry1Ac and Cry2Ab, Bollgard® crops being available upon commencement of this project it was desirable to establish if there was also root exudation of both of these proteins and to quantify the levels, given that no below ground studies existed for two gene Bt cotton.

Due to the tough nature of the tissue that comprises plant roots a sampling strategy had to be developed that would allow suitable plant tissue preparation for Cry protein extraction, whilst also permitting hight throughput of samples. A dehydration and ball mill grinding protocol was established at the ACRI, which appeared to adequately address these issues.

Cry1Ac could be quantified using the EnviroLogix Enzyme Linked Immuno System Analysis (ELISA) Cry1Ac kit, however, their Cry2 kit was based on Cry2Aa, which has only 14% cross reactive with Cry2Ab, the second Cry protein in Bollgard®. This presented a major hurdle and was overcome when we were able to source SDI Cry2Ab plates through CSD. Collaboration within the Entomology group at Narrabri permitted access to a standardised corn powder containing Cry2Ab (Monsanto Australia) that proved to be ideal for a standard preparation, which could be used to make the SDI plates quantitative. Development of the Cry protein extraction and the quantitative ELISA assay was then used to assess portioned Bollgard® plants over the 2004/05 season both above and below ground.

The ELISA techniques developed during the course of this project have been used by Leanne Scott and Donna Jones whilst working on a DEH project investigating persistence of Bt in soils, and

by Lauren Cave when working with Aphids under the supervision of Drs Mary Whitehouse and Lewis Wilson.

Leaf litter assessments:

The defoliation event in the cotton season results in the deposition of approximately 3.8 tonnes of leaf material onto the soil surface in a very short period of time. This leaf fall event signifies a major event, in terms of potential for Bt proteins to be released from degrading plant material into the soil, during the course of the season. In view of this and the large carbon pool that the leaf material represents we investigated the impact of this seasonal event on the surface soil and leaf litter microbial communities. This work represented a shift from our rhizosphere studies, but was still focused on cotton's ability to impact upon the soil biota.

Upon defoliation events in the 2004/05 and 2005/06 season a sampling strategy was implemented. It commenced one week after defoliation when leaf material was recovered from a 20 x 20 cm^2 area under conventional and transgenic cotton from three replicated plots for each variety. Once the leaf litter was removed the soil under this area was recovered to a depth of 2 cm. This sampling strategy was repeated either weekly or fortnightly depending on rain events, which were required to stimulate the leaf litter degradation. The recovered leaf material was analysed for its C and N content and also for the fungal communities it supported. Soil was used to establish substrate induced respiration experiments (SIR), microbial biomass measurements, fungal populations size, and nematode populations size and composition. In the 2005/06 season some additional work included the development of the SIR techniques to MicroResp. MicroResp is a system of SIR that utilises several carbon substrates to stimulate respiration and assesses the soils community responsiveness to them. In doing this it allows a soil profile, similar to that developed with Biolog™, to be constructed, but removes the requirement to extract the soil microbial community prior to assessing the response. Additionally, quantitative PCR was carried out on bacterial DNA isolated from the soil samples to identify fluxes in the genes *nif*H (nitrogenase reductase) and *amo*A (ammonia monooxygenase), which are involved in nitrogen fixation and nitrification. These measurements were done in collaboration with the facilities available in the CSIRO Land and Water laboratories located at Waite campus (Dr. Steven Wakelin).

Border cell recovery and counting:

Following the heavy rain event of early December 2004, access to the field sites was not possible for many weeks. During this time we undertook a simple border cell assessment to determine if the genetic modification of the cotton cultivars was having an impact on simple crop physiological properties associated with rooting.

Border cells are produced at the root cap, are terminally differentiated, survive in the environment once sloughed from the root cap, have a distinct switch in protein expression (compared to other cells of the root), are involved in environmental sensing, interact with pathogens and nematodes, produce a suite of signal molecules, and are primary determinants in root development. In light of these functions and the importance of border cells in determining how the plant 'sees' the soil environment, changes in them or to them might represent a significant observation.

Cotton seeds from a number of related and unrelated varieties were obtained from Dr Warwick Stiller and surface sterilised with a three minute wash in a 10% (v/v) bleach (4% active chlorine) and 50% (v/v) ethanol followed by washing with sterile water. Sterilised seeds (~20 per dish for each cultivar in three replicates) were transferred to a moist filter paper in an inverted Petri dish lid, the dishes were seeled with Parafilm and incubated at 28°C for 72 hours. Germinated seedlings were recovered and border cells from individual seedlings liberated into one mL of water or phosphate buffer by soaking for 5 minutes, flowed by agitation with repeated (~5) aspirations using a 200 µL pipette. The seedling was removed and the border cells in the wash counted using a Sedgwick Rafter counting chamber under the microscope (x120).

Comparisons were made between elite varieties, transgenic donors, transgenic offspring and several other cultivars that have been identified as having border cell properties of potential interest. Finally border cell numbers were compared to F and V rank data from CSD, to establish if a relationship between border cells and disease resistance existed.

Results

3. Detail and discuss the results for each objective including the statistical analysis of results.

Results from the various microbial assessments on population size, diversity and function under GM and non-GM cotton did not indicate any significant, constant or reproducible differences. In light of this, it would appear that GM cotton has no impact, deleterious or beneficial upon the soil microbial community when compared to its conventional counterparts.

It should be remembered though that the results obtained and interrogated over the course of this project are limited by the current knowledge base on soil microbiology and the technologies available at this time. There is therefore potential for GM technologies to have impacts upon the soil microbiota in ways that we are currently unable to assess or ascertain from the techniques available.

In all experiments Analysis of Variance (ANOVA) was used as the preferred method of statistical analysis. The normality of each data set was therefore questioned and where it was found to deviate from a normal distribution transformation was undertaken. Analysis of the $\log_{10}$ of the recorded data sets was normally sufficient to normalise data distribution. Once the data was normalised, blocking was used to ensure there were no field plot location interactions, measurements were assessed on individual parameters (e.g. cultivar, GM and non-GM, Bt, selective media, field) and also in combinations of the measured parameters, if interactions were thought to be likely.

A tabulated representation of the data has been included and in some instances the data has been graphed to aid with interpretation of the data sets. Significant difference or lsd values, based on 95% confidence intervals, have been included to indicate where significantly different events were recorded.

Bacterial:

Bacterial recovery was only carried out over the first two seasons due to no significant differences between cultivars being observed (tables 1 & 2). Between seasons there was a difference in recovered numbers of bacteria (figure 1), but as to whether this was a result of environment or a change in field is unknown. In the second season, a large rainfall event in December appeared to contribute to a reduction in bacterial recovery for this period (figure 2), however, the biomass recorded at the same time appeared to signify increase (figure 3). Reasons and the significance of this event are unknown, but study of flooding or waterlogging events from a microbial perspective would answer these questions. A likely caused for the increase in biomass, without recovery of larger populations of aerobic bacteria, could be due to an increase in anaerobic bacteria as waterlogging increased the number of oxygen deprived aggregates.

Table 1: Comparisons of total bacterial populations recovered from rhizosphere soil on three selective media. The mean and standard error for each cultivar are given based on assessment of soil from four plants. Media choice caused a significant difference in recovery for the 491 and 1779 day degree samples, but no GM to conventional difference was observed.

		media					
		PSA		1/10 TSA		Copio	
day degrees	Plant	mean	SE	mean	SE	mean	SE
491	189	1.5E+05	3.2E+04	1.3E+10	5.7E+08	8.6E+09	4.9E+08
	189 RR	1.3E+05	6.8E+04	1.2E+10	1.3E+09	9.1E+09	2.9E+09
	289 i	1.7E+05	2.7E+04	1.3E+10	2.2E+09	9.7E+09	9.1E+08
	289 B	2.3E+05	4.2E+04	1.1E+10	7.8E+08	9.6E+09	7.6E+08
	289 BR	1.5E+05	5.0E+04	1.0E+10	9.2E+08	6.5E+09	2.3E+09
1265	189	8.2E+05	4.8E+05	5.6E+09	4.4E+08	4.2E+09	2.5E+09
	189 RR	4.4E+05	1.3E+05	3.7E+09	2.2E+09	1.0E+10	8.6E+09
	289 i	3.2E+07	3.1E+07	3.8E+10	1.7E+10	7.2E+10	6.9E+10
	289 B	3.1E+07	3.1E+07	3.3E+10	1.9E+10	9.8E+09	7.4E+09
	289 BR	1.6E+09	1.6E+09	9.0E+09	4.9E+09	2.7E+09	4.0E+08
1779	189	2.9E+05	1.1E+05	5.5E+09	2.1E+09	5.0E+08	3.3E+08
	189 RR	3.1E+05	1.2E+05	4.1E+09	1.3E+09	1.0E+09	6.3E+08
	289 i	8.6E+05	5.3E+05	5.7E+09	6.5E+08	2.2E+09	9.6E+08
	289 B	7.4E+05	3.0E+05	4.9E+09	1.5E+09	1.9E+09	8.2E+08
	289 BR	2.2E+05	6.9E+04	3.3E+09	8.0E+08	2.0E+09	6.8E+08

Table 2: Comparisons of total bacterial populations. Mean and standard error from four plots of each cultivar are given. Media choice caused a significant difference in recovery at 292, 506 and 1602 day degrees, but no GM to conventional difference was observed.

		Media					
		PSA		1/10 TSA		Copio	
day degrees	Plant	Mean	SE	Mean	SE	Mean	SE
292	189	4.5E+07	1.3E+07	1.2E+08	5.4E+07	2.9E+07	9.6E+06
	189 RR	8.0E+07	3.5E+07	1.3E+08	5.9E+07	6.0E+07	4.0E+07
	289 B	4.3E+07	4.3E+06	8.3E+07	3.5E+07	3.8E+07	2.0E+07
	289 BR	4.9E+07	1.2E+07	8.3E+07	2.5E+07	2.0E+07	5.0E+06
506	189	9.3E+02	5.3E+06	7.0E+05	3.7E+02	8.9E+05	1.4E+05
	189 RR	1.0E+03	6.3E+06	8.2E+05	1.2E+02	8.5E+05	1.8E+05
	289 B	1.1E+06	8.7E+06	2.2E+06	1.0E+06	3.5E+06	1.6E+06
	289 BR	3.0E+05	5.9E+06	7.6E+05	1.8E+05	3.4E+05	5.0E+04
1024	189	2.8E+06	7.3E+05	1.5E+07	3.8E+06	2.8E+06	1.0E+06
	189 RR	2.5E+06	5.1E+05	6.4E+06	2.5E+06	2.4E+06	7.9E+05
	289 B	1.1E+06	1.7E+05	3.9E+06	7.3E+05	1.1E+06	1.0E+05
	289 BR	1.3E+06	5.9E+05	1.0E+07	1.2E+06	1.5E+06	2.1E+05
1602	189	3.0E+06	9.2E+05	4.4E+06	2.0E+06	3.3E+06	9.6E+05
	189 RR	6.0E+06	1.8E+06	9.5E+06	3.1E+06	6.5E+06	2.1E+06
	289 B	2.2E+06	1.3E+06	4.5E+06	2.2E+06	3.9E+06	1.9E+06
	289 BR	3.5E+06	6.0E+05	6.2E+06	2.1E+06	4.8E+06	1.2E+06

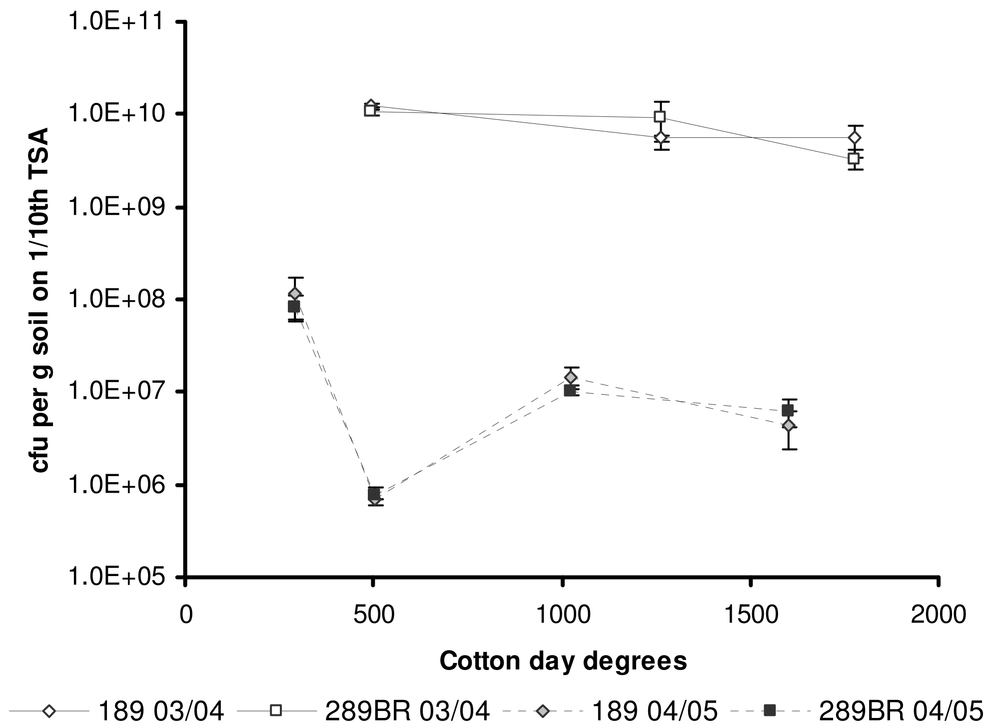


Figure 1. Comparison of bacterial colonies recovered from rhizosphere soil from Sicot 189 and Sicot 289BR on 1/10th Tryptone Soy Agar during the 2003/04 and 2004/05 seasons. Error bars represent the standard error of the means.

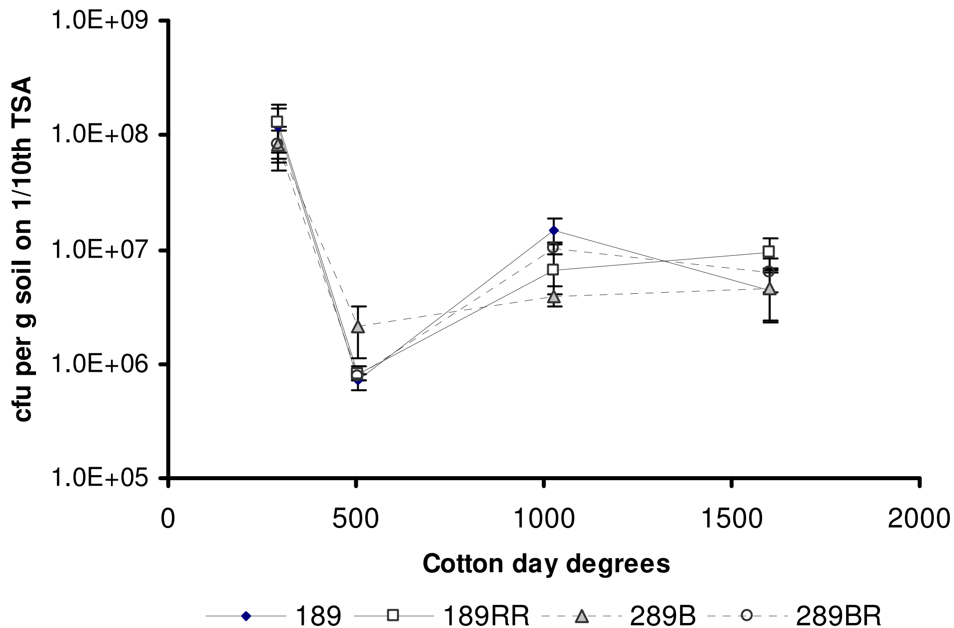


Figure 2. Comparison of bacterial colonies recovered from rhizosphere soil of four varieties on 1/10th Tryptone Soy Agar during the 2004/05 season. The depression at ~500 day degrees corresponded to the first sampling taken following a major rain event in December 2004.

Fungal:

Over the period of the project there was virtually no difference in the number of fungal colony forming units recovered from the rhizosphere soils of Sicot 189 and its transgenic derivatives grown. There were, however, few exceptions to this. Differences in the number of propagules recovered from 289BR and 289B during the 2004/05 season were recorded in two of the four sampled dates that year (table 3). During 2005/06 season the number of fungal propagules recovered from the initial soil sampling of A3 and field 14 at the ACRI showed an extremely significant (p<0.001) difference in number, regardless of media used (table 4). In the second recovery of 2005/06 71 B had significantly (p<0.001) more cfu on CD than any of the other sampled cultivars. No further differences were observed until the fifth and final sampling of the 2005/06 season field A3 still had significantly (p<0.001) more fungal propagules than field 14. This result suggests that the crop proceeding cotton, wheat in A3 and cotton in field 14 is likely to be important in determining and maintaining the fungal population throughout the subsequent season.

The observation of constancy in fungal recovery does not, however, tell us if the composition of the rhizosphere fungal community varies over the season and to address this we have started two ongoing approaches. The first of these is DGGE analysis of 18SrDNA (the fungal equivalent of bacterial 16SrDNA) and the second is community profiling based on fungal colony PCR and sequencing. The outcome and potential of these methods will continue to be assessed. Further development of these technique and reassessment of the digital database of recovered fungal cultures may be of use in the future to either the continuation of this work or other projects addressing fungal populations under cotton.

Table 3. The mean fungal cfu from rhizosphere soil recovered from four varieties grown in filed B2 at the ACRI in 2004/05. All recoveries showed a significant difference between media used. Where significant differences were observed on the same media then upper case letters have been used to indicate similar means.

		Media			
		RB		CD	
day degrees	Plant	Mean	SE	Mean	SE
292	189	13200^{B}	3400	7367	1623
	189 RR	10536^{B}	2282	7620	1114
	289 B	10880^{B}	1532	3328	355
	289 BR	27767^{A}	5312	8207	1119
significance		p=0.03		ns	
506	189	11303	2701	2231	547
	189 RR	10257	1368	2008	331
	289 B	13760	2527	3104	1331
	289 BR	13841	3714	2353	197
significance		ns		ns	
1024	189	9383	4183	5014	2313
	189 RR	9148	2367	2809	826
	289 B	10157	4207	6844	3748
	289 BR	12840	1869	2722	650
significance		ns		ns	
1602	189	3420^{AB}	758	2647^{B}	776
	189 RR	2618^{BC}	689	2474^{B}	521
	289 B	5684^{AB}	908	6414^{A}	995
	289 BR	3051^{BC}	765	2472^{B}	170
significance		p<0.05		p<0.05	

Table 4. Mean values of fungal colony forming units recovered from different varieties on Rose Bengal and Czapek-Dox agar from fields 14 and A3 at the ACRI in 2005/06. Asteric used to indicate similarity in means where a significant difference ($p<0.05$) was detected. Significant differences between the media used were again detected as were occasional differences between the fields. Comparison between fields is not indicated as different planting dates resulted in different accumulative day degree values for the two fields.

			Variety				
Field	Media	Day degrees	189	189 RR	289 B	289 BR	lsd
A3	RB	371	17975	13084	18129	26923	*ns*
		863	6001	4213	5228	11281	*ns*
		1838	9595	9022	8816	12209	*ns*
		2261	13786	14960	13082	22609*	*5728*
	CD	371	16482	12528	13164	17003	*ns*
		863	5714	2302	6310	9952	*ns*
		1838	6981	8884	6912	11399	*ns*
		2261	21559	16602	12045	14637	*ns*
14	RB	477	6688	6054	6943	7462	*ns*
		969	7420	5319	3297	5333	*ns*
		1700	5924	5469	5365	3243	*ns*
		2367	11285	9426	7409	10015	*ns*
	CD	477	4833	5658	4924	5397	*ns*
		969	8025	9564	13538	2918	*ns*
		1700		*no growth occured*			
		2367	3602	10478	3292	5844	*ns*

Respiration:

Respiration measurements as an assessment of bacterial activity in the rhizosphere were made routinely over the 2003/04 and 2004/05 seasons. The recorded values were prone to high levels of variation between samples from the same cultivars and never showed any significant difference. This was regardless of whether the measurements were from rhizosphere soil incubated in the lab or from total soil respiration measured in the field. In view of these observations and the time required to carry out titrations to establish CO_2 levels measurements were taken less often in the 2005/06 season. Again variation was a problem and no significant differences between individual cultivars were observed. However, in the final respiration assessment made in the 2005/06 season, a comparison was made between the Sicot 71 and 189 families (including both conventional and transgenic offspring), rather than individual cultivars. The Sicot71 family produced only 1.21 ug/CO_2/g/d, compared to 3.8 ug/CO_2/g/d for the Sicot 189 family. This result was significant (p=0.016) and adds further support to cultivar selection as a potential tool for the future.

The reasons behind this observation are unknown and subsequent and previous data sets did not allow interrogation of the system in this format, however, the shorter season and plant size of the 71 family, compared to 189, might have below ground implications for root development, timing of root senescence, and could provide a tool for reduced greenhouse gas emissions. Where the field based traps were used during the 2004/05 season we routinely recovered and average of 30 µg $CO_2/m^2/d$ from the 189 family of plants. This equates to the liberation of 1.6 L of CO_2 per hectare of cotton per day. Although we do not currently have a measure on the potential for cultivar selection to impact on the liberation of CO_2 extrapolation of the rhizosphere soil respiration data would suggest that this could be significantly reduced.

Biomass:

No significant differences between the grown cultivars were observed during the first two seasons of the project, however, over the course of the first year vast improvements in the reliability of our use of this technique at the ACRI were made, mainly through upgrading and improvements to equipment and sampling techniques. The results of the biomass recovery from 2005/06 resulted in significant differences being observed in the microbial biomass between rhizosphere soils from the

two fields in the initial, second and final recovery. However, difference between cultivars occurred only in A3 in the second recovery (table 5). Throughout all the experiments recorded microbial biomass was considered to be relatively low. At no time was an interaction between cultivar and field observed, and aside from the above mentioned differences, no other significant findings were recorded.

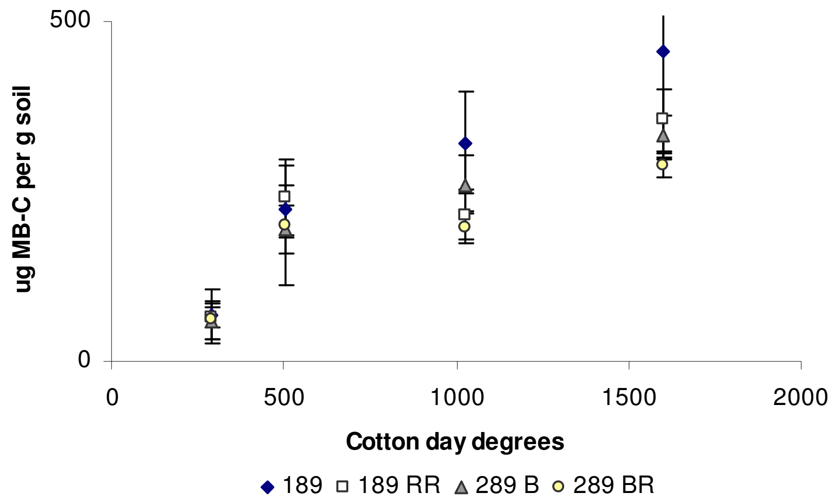


Figure 3. Mean microbial biomass of rhizosphere soil collected from cotton cultivars grown in field B2 during the 2004/05 season. Error bars represent the standard error of the mean. Of note was that culturable populations decreased around the 500 day degree sampling, but the biomass did not reflect this.

Table 5. Mean microbial biomass of rhizosphere soil in µg MB-C/g soil recovered from various cultivars in two field locations at the ACRI in the 2005/06 season. Where significant differences were observed then similar means are denoted by an identical capital letter.

		Plant				
Field	day degrees	189	189 RR	289 B	289 BR	lsd
A3	371	248	152	394	284	*ns*
	863	281^{A}	204^{BC}	242^{AB}	188^{BC}	*68*
	1838	145	169	152	158	*ns*
	2261	398	439	430	424	*ns*
14	477	518	333	448	386	*ns*
	969	170	150	141	183	*ns*
	1700	168	197	174	188	*ns*
	2367	319	339	285	303	*ns*

Ammonium Oxidisers:

Over the 2003/04 and 2004/05 seasons this assay had been prone to occasional failure, but in the 2005/06 season a full set of analysis was completed. Statistical analysis was carried out using ANOVA of the Log_{10} of the mean number returned by the MPN calculation. Log_{10} transformation was done to maintain a normal distribution to the data.

Analysis of the first recovery in the 2005/06 season indicated that there were significantly (p=0.016) more AO bacteria associated with the rhizosphere soil of 289BRR in field A3 than any of the other cultivars (table 6). By the second recovery this significant difference had been lost, but 289BRR still had a higher number of AO than other cultivars. Although not significantly different in total number a two fold difference between AO populations is associated with a difference in functional activity. By the third sampling, and in both subsequent ones, no significant difference was observed between any of the cultivars and 189RR and 289B often had the highest recorded numbers of AO from their rhizosphere soils. This change in the cultivars supporting the higher numbers of AO

bacteria could be an indication of season differences occurring. Whether this is a direct result of a change in exudation or rooting properties between cultivars is unknown, but is again basis for the types of cultivar investigation proposed in a new project with the CRDC. The data sets were also interrogated to establish if the Cry component of the transgenics might have an effect. Again no significant differences were obtained and AO populations were not consistently higher in either Cry expressing or conventional cultivars.

Table 6. Mean MPN assessed numbers of AO bacteria per g of rhizosphere soil from different cotton cultivars at different stages of developmental (day degrees) and in two fields at the ACRI, Narrabri during the 2005/06 season. Significant differences between cultivar recoveries are indicated and capital letters used to indicate similar means where a difference was recorded.

		Plant				
Field	Day degrees	189	189 RR	289 B	289 BR	significant
A3	371	1110^{B}	997^{B}	784^{B}	3931^{A}	*p=0.016*
	863	7101	4559	8616	9721	*ns*
	1838	8079	42868	23674	21690	*ns*
	2261	144462	162663	162223	64501	*ns*
14	477	753	924	1195	2715	*ns*
	969	1874	2477	1762	2378	*ns*
	1700	51887	38674	60879	236063	*ns*
	2367	34465	83073	16900	18323	*ns*

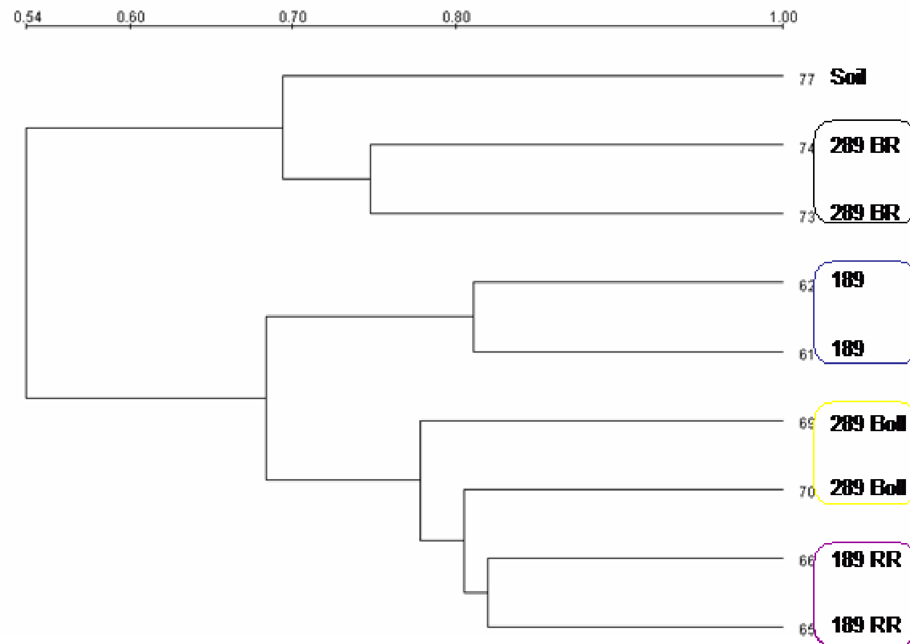
DGGE:

In the first season analysis of bacteria from the rhizosphere of glasshouse grown plants demonstrated both in Narrabri and Adelaide, from soils that had both previously supported and never supported cotton production that a clear difference was occurring in the bacterial populations identified from rhizosphere soils of different cultivars (figure 4A). These glasshouse results indicated that bacterial populations associated with Sicot 189RR and 289B rhizosphere showed closest similarity to Sicot 189 but less similar to that of Sicot 289BR. Bacterial populations associated with 289BR were less similar to the other three cultivars. DGGE results from glasshouse trial using SA soil (that is not exposed to cotton) showed similar variety based differences in bacterial populations (figure 4C). However there was no clear separation of data on rhizosphere fungal communities (figure 4D). At this stage no specific GM and non-GM component was evident as being the only major influencing the rhizosphere microfloral composition. This was made evident by the fact that different GM traits were not branching at the same level of similarity. When field analysis was undertaken using this technique the cultivar differences became less apparent (figure 4B). This was probably a result of a combination of the following factors occurring in the field as opposed to the glasshouse: changes in stresses due to temperature and watering, differences in plant physiology of field versus glasshouse plants, the flow of irrigation water down the field and through cracks within the clay, and the movement of biological organism from the bulk to rhizosphere soil. Additionally, microorganism distribution in field soils is patchy (vertically and spatially) so collection of representative samples is difficult, often resulting in higher variability in microbial assessments of field soils. In the glasshouse experiments, where plants were grown in a more uniform soil and collection of rhizosphere soil was easier, this overriding variability in microbial distribution would have been significantly reduced.

Over the 2004/05 and 2005/06 more DGGE analysis was undertaken and the scope of the work increased to include fungal populations (through 18srDNA assessments) and *Burkholderia* (through genus specific primers). These results showed no consistent differences in bacterial or *Burkholderia* diversity between fields or cultivars (figure 5). Preliminary results suggest that there was a weak separation between fields for the microbial population, but variation was greater amongst replicates than between cultivars. For *Burkholderia*, the population structure was more variable amongst replicates than between fields or cultivars.

Figure 4. Dendograms created from DGGE analysis of PCR products of 16srDNA analysis of bacteria obtained from rhizosphere soil. The clamped PCR products analysed in the dendograms are: (**A**) 16srDNA analysis of bacteria from a glasshouse trial in 2003 showing clear separation of bacterial communities by cultivar, but not being driven by transgenic components, (**B**) 16srDNA analysis of bacteria from the 2003/04 field in which the cultivar differences are less clear than the glasshouse. This difference from the glasshouse trial is probably as a result of bulk soil rhizosphere interactions and flood irrigation events.

A



B

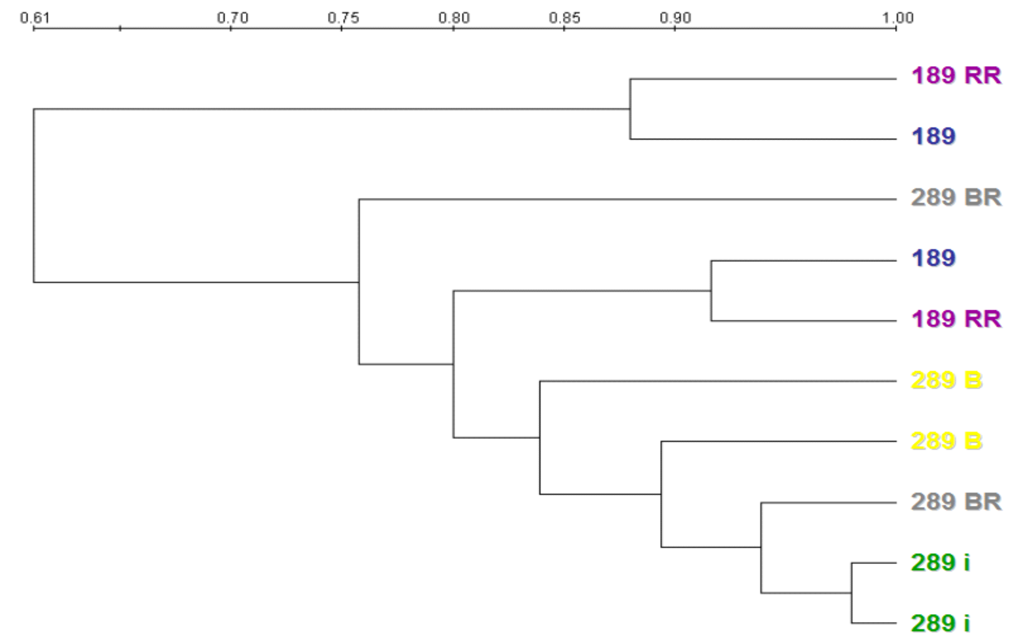
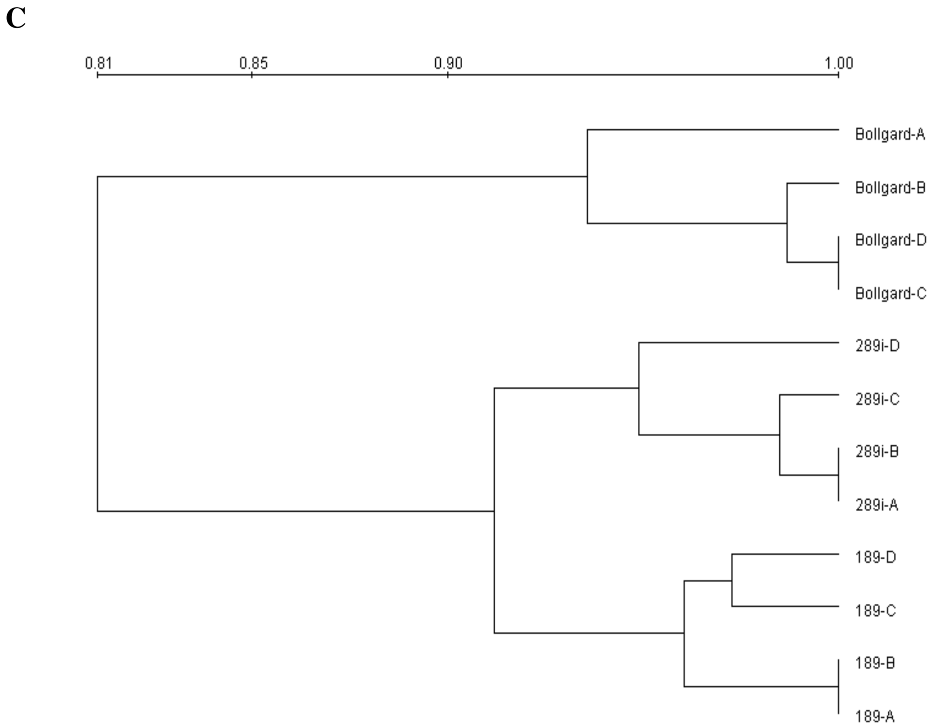


Figure 4C&D. Dendograms created from DGGE analysis of PCR products of bacterial and fungal populations obtained from rhizosphere soil from a glasshouse trial using a South Australian soil that was not previously exposed to cotton: (C) 16srDNA analysis of bacteria showing a separation of bacterial communities by cultivar, (D) 18S rDNA analysis of soil fungal communities showing no clear separation between Bt cotton and conventional varieties.

C



D

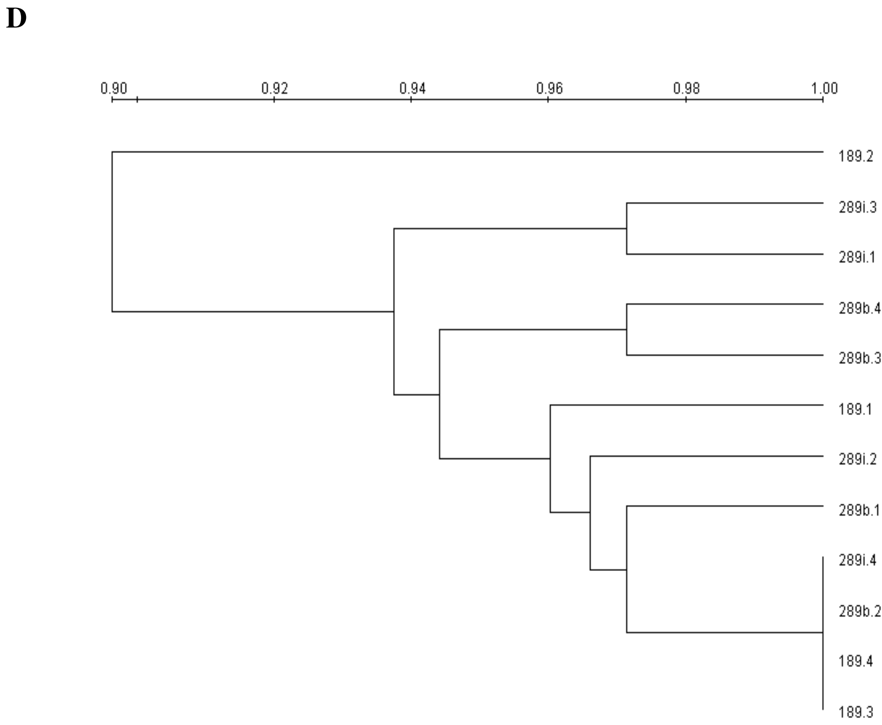
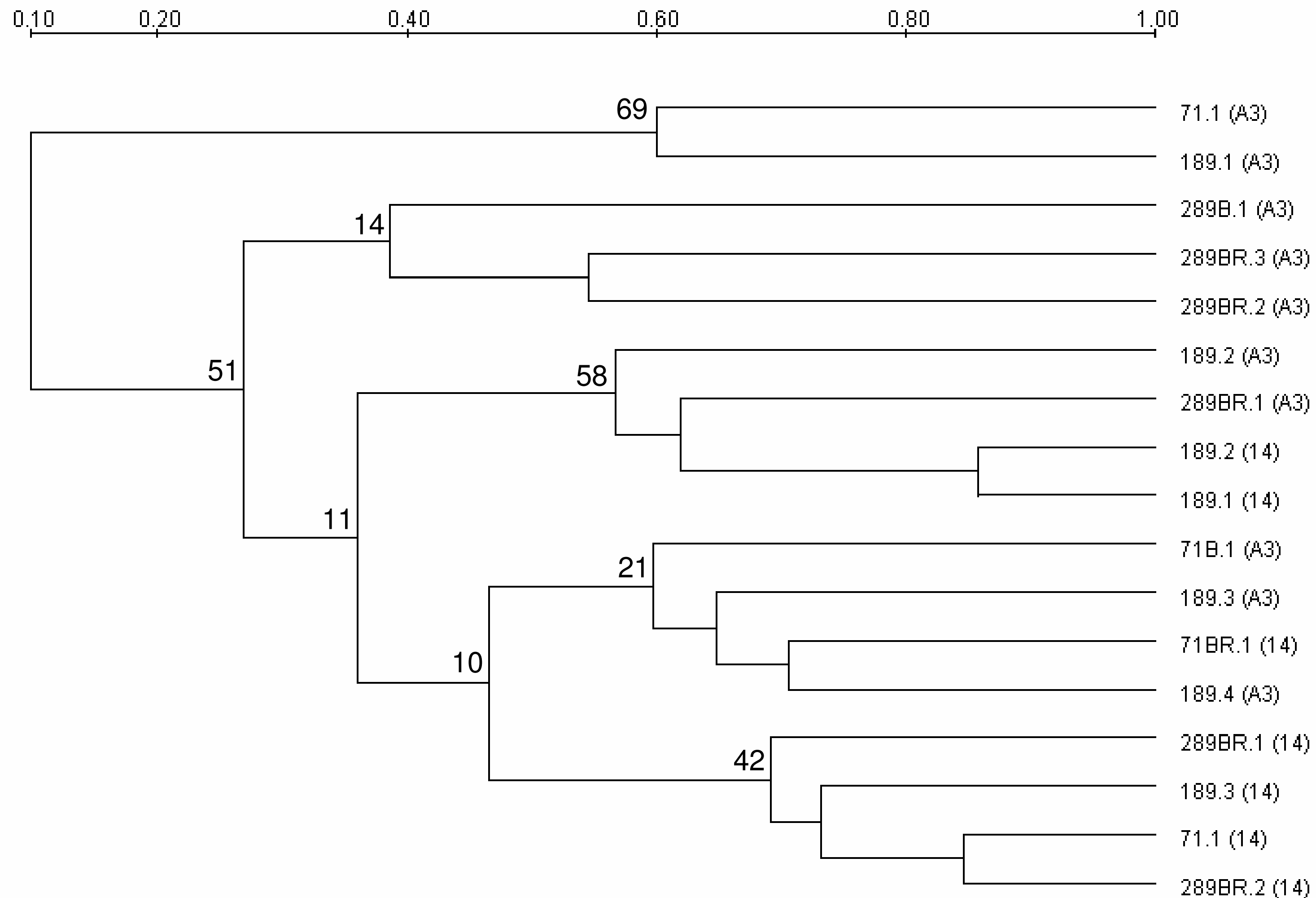


Figure 5. Dendogram created from a *Burkholderia* specific clamped PCR, assessed on a DGGE gel and analysed using Dice's UPGMA. The analysed rhizosphere soil samples were recovered during the 2005/06 season from fields at the ACRI in February 2006. Numbers indicate the variety, letters the transgenic traits or conventional status, and the number after the decimal point indicates the specific replicate. Letters and numbers in brackets indicate the field source of the sample. Interpretation of the branching indicates that there was weak separation between fields, but greater variation between cultivar replicates.



Although no GM to non-GM difference was observed and the cultivar selection of microbiota was either less clear or not apparent under field conditions there is still plenty of potential for DGGE as a powerful tool for interrogating soil microbiology. The development, fine tuning and employment of these techniques over the course of this project represented a considerable effort in terms of man hours and resources and have provided the group with considerable expertise in this area. The use of DGGE analysis as a resource in rotation and cultivar investigations will be maintained and continued.

Yield:

Over the first two seasons, when all crops were grown in fields managed as if containing conventional cotton, there was no difference in cultivar performance observed. In the final 2005/06 season differences between the sprayed and unsprayed trial sites were expected. Field 14, the unsprayed paddock, yielded significantly less cotton than A3. Additionally, further analysis of these results indicated that there was a cultivar difference in yield within the Sicot 189 family. The Bollgard crops faired better in both sites, but the performance of 189 RR was surprising, given that it faired almost as well as the insecticidal transgenics within the unsprayed field environment. The Sicot 71 family was excluded from comparison in this analysis due to the low number of replicates available, but yielded 7.7, 8.9 and 6.6 ba/h for 71, 71 B and 71 BR, respectively over both field sites.

Table 7. Mean yields converted to ba/a, assuming 40% lint from the replicated 7 m plots, for the Sicot 189 'family' in each season of the project. All field trials were managed as if conventional cotton and spray regimes were implemented to reflect insect pressure, with the exception of field 14 in 2005/06, which was an unsprayed field.

	Plant	Mean yield Ba/a	SE	Significance
A3				
2003/04	189	4.1	0.1	
sprayed	189 RR	3.7	0.1	
	289 i	4.4	0.0	
	289 B	4.1	0.2	
	289 BR	3.7	0.3	*ns*
B2				
2004/05	189	4.4	0.3	
sprayed	189 RR	4.0	0.3	
	289 B	4.6	0.1	
	289 BR	4.7	0.2	*ns*
A3				
2005/06	189	4.4	0.1	
sprayed	189 RR	4.5	0.2	
	289 B	5.1	0.2	
	289 BR	5.1	0.2	*ns*
14 2005/06	189	2.16^{A}	0.3	
unsprayed	189 RR	2.58^{B}	0.2	
	289 B	3.04^{B}	0.3	
	289 BR	3.28^{B}	0.3	*p=0.08*

Quantitative ELISA:

We initially undertook an ELISA based assessment of expression levels of Cry1Ac in the roots of Sicot 289 Bollgard®II (289B) and Sicot 289 Bollgard®II Roundup Ready (289BR) plants grown in the glasshouse versus the field. In the roots of these plants Cry1Ac expression averaged 0.03 µg/g for the glasshouse, compared to 0.14 µg/g for the same Bt- cotton varieties grown in the field.

Given the apparent significant effect of glasshouse conditions on Cry expression, in the 2004/05 season we quantified Cry1Ac and Cry2Ab in field grown Sicot 289B and 289BR for both above ground and below ground plant tissues. Both Cry proteins were detected in the root material.

Significant differences among Cry1Ac and Cry2Ab and seasonal changes of whole plant levels of expression were also observed, with Cry1Ac levels remaining relatively constant at an average of 19.1 µg/g whilst Cry2Ab levels decreased over time and averaged only 11.5 µg/g. Analysis of whole plant expression levels (including the roots), plant stand densities, and aspects of crop management, estimated that levels of Cry1Ac and Cry2Ab incorporated into the soil at the end of the season were 0.26 and 0.16 µg/g soil, respectively.

Leaf litter assessments:

Cotton plants contribute carbon inputs to the soil through roots (root turnover and rhizodeposition), leaf residues and stubble. While the contribution from roots occurs throughout the growing season (smaller amounts but over long period of time), carbon inputs from leaf residues and stubble are made closer to or after harvest. Leaf drop, following defoliation, is a single major event when a large dose of carbon inputs come in contact with soil biota. Results from our previous work indicated significant increases in microbial activity and populations of bacteria and fungi in soil directly below (0-2.5 cm surface soil) fallen and decomposing leaves probably due to the easily available carbon inputs from leaf material. During the 2004/05 and 2005/06 cotton season we measured the composition of overall microbial community, soil fungi and functional groups important for nutrient cycling, with more intensive study applied in the 2005/06 season. In general, community level physiological profiles have given useful results about the dynamics of soil microbial communities as influenced by leaf residue. The principal component analysis (PCA) plot of data on the microbial communities involved in C cycling (figure 6) indicates significant discrimination between different cotton varieties at both represented sampling times. The first two principle components accounted for ~70% of the total variation. PCA factor 1 (pc1) separated the 71B and 289BR communities from their counterpart conventional varieties. Differences between varieties separated by pc2 factor were smaller and not significant in the early sampling. Similarly PCA results of soil fungal communities (figure 7) indicated differences between varieties; pc1 factor accounting for 43% of variation and differences between GM and non-GM varieties were mostly accounted by PCA factor 1 only.

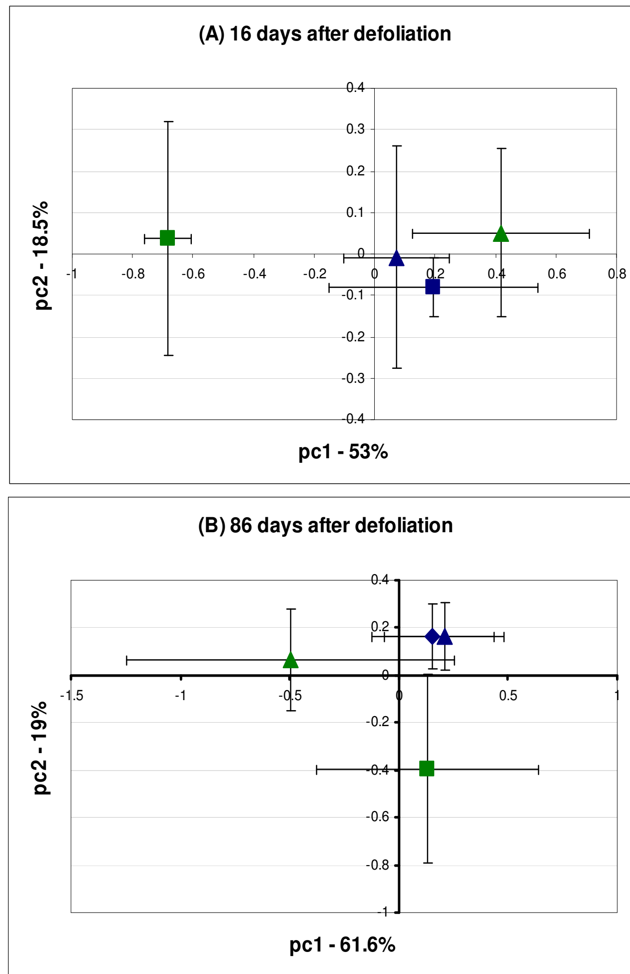


Figure 6. A PCA graph of the data on the composition of soil microbial communities associated with decomposing leaf residues measured in the 2005/06 season and based on carbon substrate utilization profiles. (A) 16 days after defoliation and (B) 86 days after defoliation. Blue squares - 71; green squares - 71B; Blue triangles - 189; green triangles - 289BR.

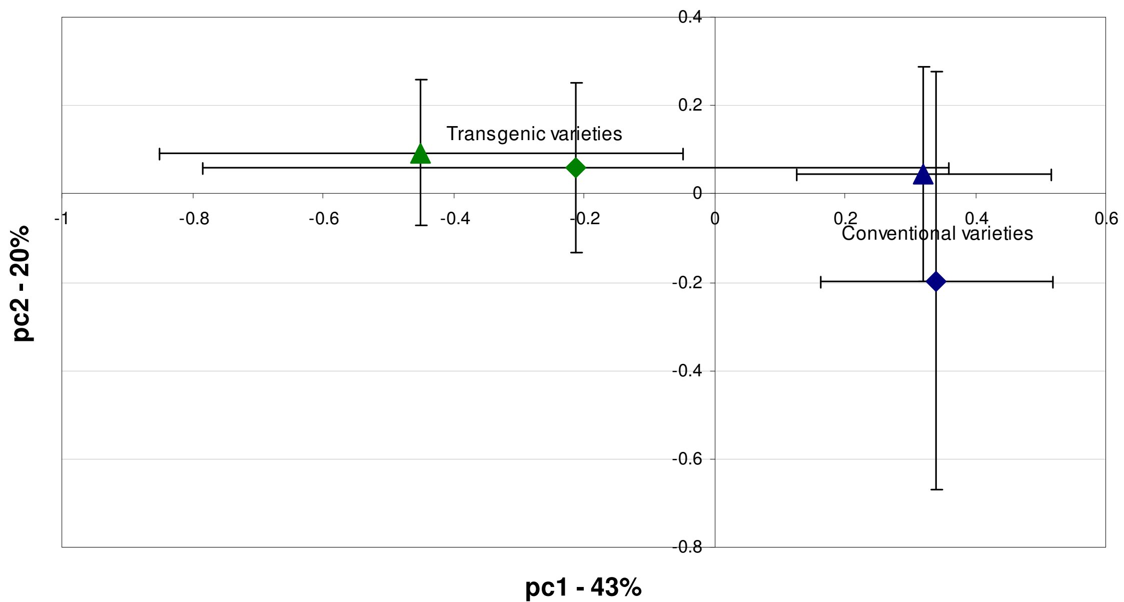


Figure 7. Diversity of fungal communities associated with soil directly below the cotton leaves after defoliation determined using carbon substrate utilization profiles – BIOLOG FF (2005/06 season). Error bars represent standard error of means.

Fungal populations within the soil were assessed in the 2004/05 and 2005/06 seasons. There was no significant difference observed in these total population numbers in response to the cotton variety responsible for the leaf litter fall, but as the soil was monitored post defoliation an increase in the total fungal population occurred (tables 8&9), presumably as the carbon from the leaf litter was taken up into the biological component of the soil. In the 2005/06 season the leaf litter fungal populations were also investigated. The fungal leaf litter population also showed no signs of variation between cultivar source, but unlike the soil population it remained relatively stable, although much higher than the underlying soil (Table 9). The increase in the soil's fungal community numbers following defoliation is perhaps of no surprise, but the organisms of which it is comprised and the fate of this biota during non-crop period is currently unknown and, like so much soil biology during off-season, warrants further investigation.

Table 8. Post defoliation changes in the total soil fungal community recovered from the top 2 cm of soil under defoliated leaf material in 2005. The means for each cultivar are given and the media used are Czapek-Dox (CD) and Rose Bengal (RB).

2005		**Days after**	**Cultivar**				
Sample	**Media**	**defoliation**	**189**	**189 RR**	**289 B**	**289 BR**	Significant
Soil	CD	7	5135	6351	5096	7013	*ns*
		21	3479	2526	2397	3605	*ns*
		98	28822	22140	25042	31826	*ns*
	RB	7	2608	4404	4537	2781	*ns*
		21	5897	3217	4582	5862	*ns*
		98	23300	21188	14408	35920	*ns*

Table 9. Post defoliation changes in the total soil and leaf litter fungal community recovered from the top 2 cm of soil under defoliated leaf material in 2006. Recovery was on Czapek-Dox (CD) and Rose Bengal (RB) media and the means for each cultivar assayed are presented.

2006		**Days after**	**Cultivar**				
Sample	**Media**	**defoliation**	**71**	**71BR**	**189**	**289BR**	Significant
Leaf Litter	CD	16	2534061	1799679	4406731	1323266	*ns*
		30	1301354	3932786	1784620	2540956	*ns*
		86	*None available*				
Soil	CD	16	14274	17577	13130	14867	*ns*
		30	19742	26829	33755	24956	*ns*
		86	43208	41406	37954	70562	*ns*
	RB	16	20677	21229	18840	18831	*ns*
		30	57927	60750	74848	53251	*ns*
		86	104804	122377	129672	140646	*ns*

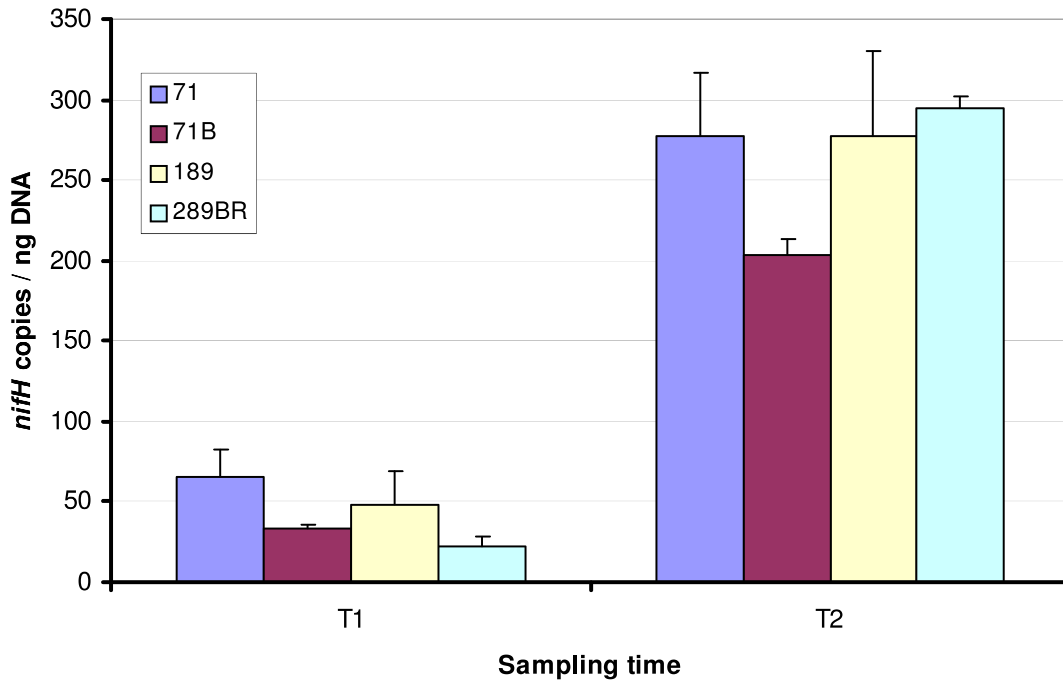
Microbial biomass was also assessed over the two years of the defoliation experiment, again for soil collected directly under fallen leaf material to a depth of no more than 2 cm. As with the fungal recovery there was no observed significant difference in microbial biomass from soil collected from under different cultivars, however, differences were observed over the time course of each experiment (table 10). These were also noted to differ between the two years and were probably a reflection of the different rain fall events and available moisture between the two years as well as a function of the sampling times.

Table 10. The mean microbial biomass carbon, as assessed by the ninhydrin method, for a number of soils collected form under different cultivars post defoliation (DAD = Days After Defoliation) toward the end of the 2004/05 (2005) and 2005/06 (2006) seasons. Where significant differences are indicated similar means are indicated with identical capital letters.

		DAD		
2005	**7**	**21**	**112**	significant
Mean	188.2^{B}	207^{A}	133.8^{C}	lsd=27.1
Variety				
189	201.2	181.6	149.8	
189 RR	174.9	226.2	133.8	
289 B	187.7	200.6	121.9	
289 BR	189.1	219.7	129.7	
significant	*ns*	*ns*	*ns*	

		DAD		
2006	**16**	**30**	**86**	significant
Mean	198.5^{B}	292.1^{A}	267.3^{A}	lsd=49.5
Variety				
189	163.4	287.8	214.5	
289BR	215.3	282.1	259.1	
71	173.9	279.3	286.7	
71B	241.4	319	309	
significant	*ns*	*ns*	*ns*	

Results on the functional groups of microbial communities involved in nitrification (*amo*A) and nitrogen fixation (*nif*H), shown in figure 8, indicate significant increase in both communities from day 16 to day 86 after defoliation reflecting the positive impact of C inputs from leaf residues on these communities. For both communities, differences between varieties were smaller than the temporal differences. While there was no difference in *nif*H levels between 189 and 289BR at both sampling times, a significant difference between 71 and 71B can be seen at day 86. Differences of this kind between GM and non-GM varieties were not seen with *amo*A levels.



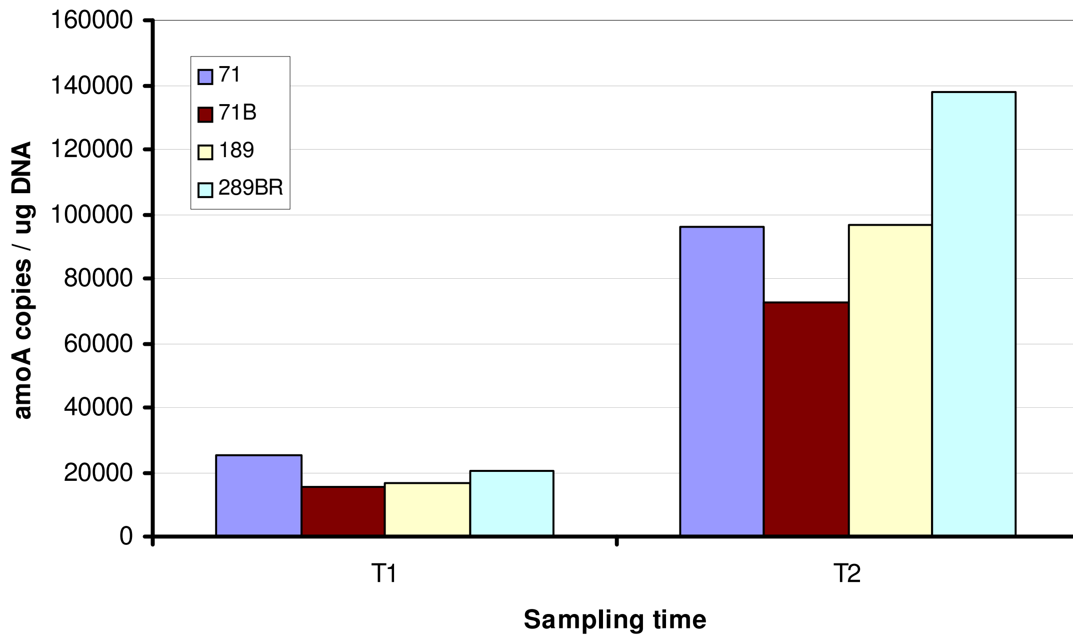


Figure 8. Changes in the levels of (A) N-fixing bacteria and (B) nitrifying organisms in soils under cotton leaves measured using quantitative PCR (Q-PCR) of *nif*H and *amo*A genes. Sampling times T1 and T2 represent 16 and 86 days after defoliant application.

Nematode populations were also monitored in the soil directly under the leaf litter, although cost restricted a detailed analysis until the 2005/06 season. As with the fungal community there was a dramatic response in the nematode populations to the carbon input from the leaf litter into the system and, just like the fungi, this difference did not appear to be influenced by the cultivar.

In the only assessment made in 2004/05, at 14 weeks post defoliation, there were two observations of note. The first of these was that the nematode population was very small with less than 0.7 nematodes per g soil recovered, but the population was quite diverse (figure 9). The second observation was that the numbers of nematodes increased significantly (*p=0.015*) as the sampled plot locations got nearer to the tail drain (figure 10). Reasons for these observations are unknown, but it could be speculated that the almost complete degradation of the leaf litter had resulted in a decline in nematode numbers and that the increase down the field was a result of water movement with irrigations carrying nutrients or food down the field.

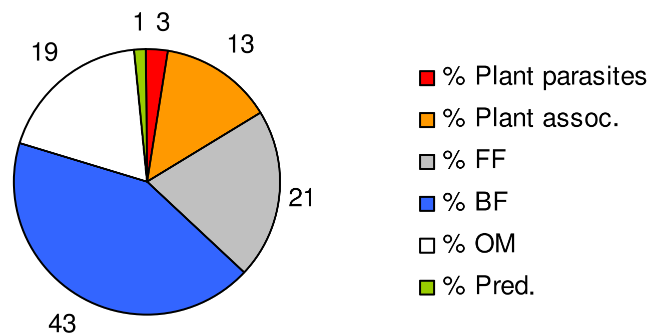


Figure 9. The nematode population of field B2 as sampled in July 2005, 14 weeks post defoliation, presented as percentages of each trophic group. The total populations recovered were small with less than 0.7 nematodes per g soil (dry weight equivalent) recovered.

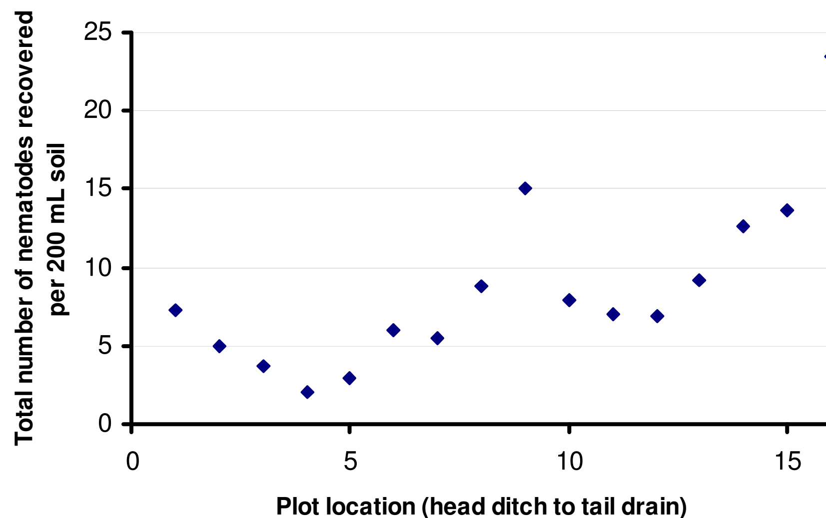


Figure 10. The total number of nematodes per 200 mL soil sample recovered from field B2 14 weeks post defoliation. The increase in numbers as the plots moved from the head ditch (plot 1) to the tail drain (plot 16) was significant.

In the 2005/06 season samples were taken with each of the soil and leaf litter recoveries in an attempt to better understand how the nematodes were responding to the leaf litter drop and also to assess if there was a cultivar difference. Again no cultivar difference was observed and the increase in distribution down the field, as observed the previous year, was not seen. The overall population composition was also somewhat reduced, but the total number of recovered nematode increased, compared to the previous season (figure 11). Reasons for this difference in populations and their

composition between the two years are not know, but could have been a reflection of the change in field to A3 or a difference in rotation. Again, as with the fungal work, the fate of these nematodes in terms of where they go, what they represent and their significance to the cropping system is poorly understood and warrants further investigation.

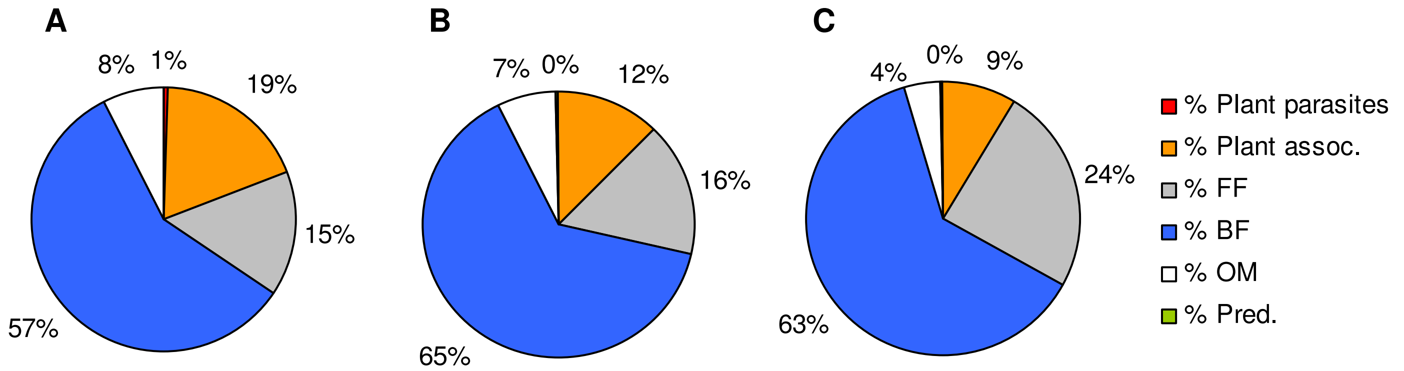


Figure 11. Percentage contribution of various nematode trophic groups to the nematode population recovered from A3 in 2006 at 7 (A), 30 (B) and 86 (C) days after defoliation. The size of the total nematode populations in each of these assessments differed with a mean number of nematodes per g of soil of 4.3, 4.4 and 25.9 for A, B and C, respectively.

Populations and activity of microbial communities in Australian agricultural soils known to be of low fertility do respond to addition of carbon inputs. Our results suggest that carbon inputs from leaf residues following defoliation significantly influence the populations and composition of general soil microbial community and composition of soil fungi. It seems that the nature of influence may be different for different cotton varieties. The influence of the sudden explosion of biological activity and changes to various groups of microbial communities on important soil functions such as nutrient (e.g. N, P), survival of pathogenic fungi etc are not known. More importantly, how we can make use of this period of high microbial activities to the benefit of the cotton farming system is not known. As indicated before, recent molecular techniques provide us with great opportunity to study the changes in important functional groups of soil microbiota and in this study we only measured populations of two functional groups. Future research on the detailed analysis of various functional groups of microbial communities, e.g., N mineralization, denitrification, green house gas emission, decomposition, microbes capable of biocontrol etc is warranted.

Border cell recovery and counting:

Border cells were recovered from each assessed cultivar, however, only Sicot 189 was observed to be producing the 10,000 border cells per root tip previously reported for *Gossypium*. Most other transgenic and transgenic donor varieties were producing a lot less border cells, typically in the range of 2,000 – 5,000. This decrease in border cells did not appear to be entirely due to the transgenic insertion event, but was possibly a result of the backcrossing involved in the variety construction and selection process. Although no associated problem with a decrease in border cell number has been observed in these varieties, it was noted border cell number in the 2,000 – 6,000 range explained 30% of the F-rank value. Border cells are therefore having some role in resistance toward this fungus, however, further evidence of this is required and the findings have been discussed with Dr Stephen Allen (CSD) and Mr Chris Anderson (NSW DPI) to foster collaboration in this area.

Outcomes

4. Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

This research contributed to CRDC Output 1 – "Sustainability of Natural Resources" (specifically the "Soils" program) because the cotton rhizosphere microfloral populations are key participants in the maintenance of long-term soil quality and health. Demonstration of the lack of difference in the rhizosphere microflora associated with GM and non-GM cotton indicated that adoption of the GM technology has not put this balance at risk.

Grower awareness of soil health as an issue appears to have grown since the inception of this project following the 2001 'Soil Health Workshop' at Narrabri. This has been demonstrated by the number of industry talks in this area both requested and given by the researchers over the life of the project. Additionally, the appointment of Dr. Knox at Narrabri increased the capacity for research in the biological disciplines (namely soil biodiversity and biological health) associated with cotton farming, as was recognised as a requirement at the same 2001 meeting.

In addition to the annual reports the project has produced several scientific publications, industry related articles, and presented data at a range of pertinent meetings.

Student training and collaboration:

A CRC summer scholarship was proposed and undertaken in collaboration with Nilantha Hulugalle (NSW DPI) and Daniel Tan (Uni of Sydney). The student, Nick Luelf, developed the project into an honours thesis and presented the work at the Agronomy Conference in Perth, August 2006. During the life of the project we have also undertaken student support in a BHP Billiton and two CSIRO Student Research Scheme projects. These have allowed students from the local high schools to undertake research at the ACRI and established a further link between the undertaken research and the community.

5. Please describe any:-
   a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);

No patents or commercialisation was developed from the outcomes of this project.

   b) other information developed from research (eg discoveries in methodology, equipment design, etc.); and

- A method for cotton rhizosphere soil sampling was established and interrogated and found to be suited to the methods of study described in this report.
- Quantitative Cry2Ab ELISA methodology was developed from existing research tools (SDI Cry2Ab plates) and through access to suitable standards.
- The suite of available microbial techniques at the ACRI was expanded and developed.
- A number of molecular methods to measure the diversity of various microbial groups were successfully adopted for cotton soils.

   c) required changes to the Intellectual Property register.

None.

Conclusion

6. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?

The take home messages from this research are:

- Glasshouse and field evaluations of transgenic cotton are not comparable due to different physiological and environmental conditions both experienced and expressed by the plant when grown under these two systems.
- In field experiments our assessment of rhizosphere microbial population size, diversity and function does not appear to be influenced by the presence or absence of GM traits currently available in cotton. However, in glasshouse experiments, significant differences in composition of rhizosphere bacteria were observed between GM and non-GM cotton varieties.
- Within both glasshouse and field work there has been some clear experimental evidence generated that cotton cultivars can have a significant impact upon the microbial community. The extent of this and its potential for management has formed the basis of a new project with the CRDC.
- Carbon inputs from leaf residues following defoliation significantly influence the populations and composition of general soil microbial community and composition of soil fungi, and differences between GM and non-GM varieties were observed.
- Soil biology is exceptional diverse and predominantly microscopic. With the tools available today we are still trying to unlock many of its secrets. Work, similar to that outlined in this project and report is essential if we are to ever capitalise on the soil's natural functions, as provided by this biota, within an agricultural system.

Extension Opportunities

7. Detail a plan for the activities or other steps that may be taken:
   (a) to further develop or to exploit the project technology.
   Although no new technology was developed, the increased suite of microbial assays available at the ACRI has already been utilised. Continued collaboration and the fostering of new research relationships to capitalise on these capabilities are envisaged.

   (b) for the future presentation and dissemination of the project outcomes.
   Interest in soil biology within the cotton industry is still high. Making staff and research outcomes available for presentation at industry relevant events will remain a focus of the group. Dissemination of further particulars of the research is proposed in Cotton Grower articles and scientific papers (see 8. below).

   (c) for future research.
   (1) Varietal differences were observed. A new project to to assess their impact on certain aspects and members of the soil microbial community has been proposed and received funding.
   (2) Functional impacts of C inputs from leaf residues on microbial communities and essential microbial functions during non-crop period and potential consequences to next crop are not known and require future investigation.

9. A. List the publications arising from the research project and/or a publication plan.
(NB: Where possible, please provide a copy of any publication/s)

Published documentation is available from the researchers and collaborators upon request.
The following articles are proposed to be addressed and written over the coming year.
Proposed Cotton Grower articles on:

- Mycorrhizal association with Gm and non-GM cotton – Spot the difference?
- Nematodes in Australian cotton

Proposed scientific papers:

- Soil microbial populations under GM and non-GM Australian cotton
- The impact of defoliation on soil biota from a GM and non-GM perspective
- Mycorrhizal association with Gm and non-GM cotton

Currently under internal review:

- **Oliver G. G. Knox**, **Gupta V.S.R. Vadakattu**, **Grant N. Roberts** and Sharon J. Downes. Quantitative seasonal expression of Cry1Ac and Cry2Ab in field grown Bt-cotton in Australia
- **Oliver G.G. Knox**, Chris M.T. Anderson, David B. Nehl and **Vadakattu V.S.R. Gupta**. Observation of *Tylenchorhynchus ewingi* in association with cotton in Australia (submitted to Australasian Plant Disease Notes)

Peer reviewed publications:

- **Knox, O.G.G., Gupta, V.V.S.R.**, Nehl, D.B. and Stiller, W.N. Differences in border cell number and constitutive root tip expression of Cry proteins in transgenic cotton. *Euphytica* (accepted August 2006)
- **Knox, O.G.G.,** Constable G.A., Pyke B. and **Gupta V.S.R.V.**(2006) Environmental impact of conventional and Bt insecticidal cotton expressing one and two Cry genes in Australia. *Australian Journal of Agricultural Research* **57**(5) 501-509.
- **Knox, O.G.G.**, Anderson, C.M.T., Allen, S.J. and Nehl, D.B. (2006) *Helicotylenchus dihystera* in Australian cotton roots. *Australasian Plant Pathology* **35**, 287-288.

Book chapters and refereed proceedings:

- Nehl, D.B. and **Knox, O.G.G.** in *Soil Biology, Volume 7. Microbial Activity in the Rhizosphere* pp.89-119 (eds. K.G. Mukerji, C. Manoharachary, J. Singh) Springer-Verlag Berlin Heidelberg 2006
- 13th Australian Agronomy Conference. Luelf, N., Tan, D., Hulugalle, N., **Knox, O.**, Weaver, T. and Field, D. (September 2006) Root turnover and microbial activity in cotton farming systems
- 6th Pacific Rim Conference. **Knox, O.G.G.** and **Gupta, V.V.S.R.** (November 2005) 'Evaluation of border cell number and Cry expression from root tips of *Gossypium hirsutum*'.

Non refereed papers and meeting proceedings:

- ACGRA 13th Australian Cotton Conference. Seymour, N., **Knox, O**. and **Gupta, V**. (August 2006) 'Understanding Soil Biology'.
- ACGRA 13th Australian Cotton Conference. **Knox, O. and Gupta V.** (August 2006) 'Variation in microbial community and function within cotton fields'
- *Cotton Grower*. **Oliver Knox**, Ian Rochester, **Gupta Vadakattu** and Louise Lawrence. (August 2006) 'Composting in Australian cotton production'.
- Cotton Consultants Certified Members Meeting. **Knox, O.** (July 2006) 'Soils Research'.
- 2006 Lower Namoi Field Day Book. **Knox, O.G.G.**, Anderson, C.M.T., Allen, S.J. and Nehl, D.B. (February 2006) 'Nematodes in Australian Cotton'.
- Australasian Plant Pathology Society Conference. Anderson, C.M.T., **Knox, O.G.G.**, Nehl, D.B. and Allen, S.J. (September 2005) 'First record of the root lesion nematode *Helicotylenchus dihystera* in cotton in Australian'.
- FUSCOM PLUS. **Knox, O.G.G.** and Anderson, C.M.T. (June 2005) 'Interactions of Verticillium and nematodes: a very early Australian perspective.'
- Cotton Consultants Australia Inc. AGM. **Knox, O.G.G.**, **Gupta, V.V.S.R.**, Middgely, D.J., Loke, S., McGee, P.A., Wang, B., Nehl, D., Anderson, C.M.T., Harvey, J. and Baker, G. (May 2005) 'Underground Cotton Research'.
- Lower Namoi 2005 Field Day Book. **Knox, O.G.G.** and **Gupta, V.V.S.R.** (March 2005) 'Conventional and genetically modified cottons' influence on microbial populations'.

- Australian Cotton Cooperative Research Centre, Cotton information sheet. **Knox, O.G.G.**, **Gupta, V.V.S.R.**, Seymour, N. Rourke, K.L., Whiffen, L.K., Middgely, D.J. and McGee, P.A. (November 2004) 'Soil Biology: What's hiding in the ground?'
- CRDC and Cotton CRC Farming Systems Forum. **Knox, O.G.G.**, **Gupta, V.V.S.R.**, Middgely, D.J., Loke, S., McGee, P.A., Wang, B., Nehl, D., Anderson, C.M.T., Harvey, J. and Baker, G. (November 2004) 'Underground soil research and soil health'.
- ACGRA 12th Australian Cotton Conference. **Gupta, V.V.S.R.**, Watson, S.K. and **Knox, O.G.G.** (August 2004). 'Below ground production of Bt-protein by Genetically Modified cotton varieties'.
- ACGRA 12th Australian Cotton Conference. **Knox, O.G.G.**, **Gupta, V.V.S.R.**, Seymour, N. Rourke, K.L., Whiffen, L.K., Middgely, D.J. and McGee, P.A. (August 2004) 'Soil Biology: What's hiding in the ground?'
- Cotton Consultants Australia Inc. AGM. **Knox, O.G.G.**, **Gupta, V.V.S.R.** and **Roberts, G.N.** (May 2004) 'Cotton soil ecosystems: present views and future trends.'

B. Have you developed any online resources and what is the website address?

Access to the soil information sheet developed from this project is available at http://www.cotton.crc.org.au/Assets/PDFFiles/Soil/Soilbiol.pdf.

Part 4 – Final Report Executive Summary

Variety based significant differences in rhizosphere microbiota were observed but no significant differences in rhizosphere microbial communities under field grown GM and non-GM cotton varieties

Experiments to establish if the use of GM cotton has an impact on the soil microbes that grow in association with the plant roots has shown that there are no differences in number or function when compared to non-GM conventional cotton under field conditions. However, varietal differences were observed in both the field and glasshouse.

Experiments were run in cotton field trials over three seasons at the Australian Cotton Research Institute, near Narrabri, NSW. Rhizosphere soil, the soil that makes contact with the root and is directly influenced by the plant, was routinely sampled. Bacteria and fungi were recovered from this soil using traditional cultural techniques on several selective agar media and found not to differ in numbers between GM and non-GM. Experiments to investigate activity (measured as respiration) and amount of microbes (assessed as biomass) also showed no differences between the GM and non-GM plants under our experimental conditions. A desk top environmental impact assessment of insecticide use, made during the project, also indicated that GM cotton is less environmentally damaging than it's conventional counterpart and their associated pesticide usage. With these two considerations in mind, GM cotton would appear to be the more sustainable and less environmentally harmful option of cotton production currently available in Australia.

Despite no significant GM to non-GM differences being observed in microbial biomass and activity, cotton varietal differences were noted during the course of the project. Molecular work, using a technique known as DGGE to produce a 'fingerprint' of microbial communities, produced evidence that varieties were selecting specific microbial populations in association with their roots. This was apparent from glasshouse trials conducted in Narrabri and Adelaide, on both cotton and non-cotton soils. Lack of consistent differences under field conditions could be attributed to stresses due to environmental factors such as temperature, water availability and plant physiology differences between glasshouse and field.

Varietal differences were again noted when border cells produced by cotton cultivars were assessed. Border cells are produced at root tips and are involved in environmental sensing by the plant. Border cell numbers were much lower in many of the currently available GM and non-GM cotton varieties. Tested varieties produced between 2000 to 12000 border cells per root tip. Reasons

for this varietal difference were unclear, but there was evidence that border cell number plays a role in Fusarium resistance.

Assessment of the impact of leaf drop following defoliation indicated that this sudden carbon deposit onto the soil significantly/dramatically increased adjacent soil biota. No consistent varietal differences in the microbial biomass levels were observed, however, the composition of microbial communities associated with decomposing leaf residues was influenced by variety. This work does raise questions regarding the functional significance of this explosion of biota and if management could alter or better utilise this process. Future research to fill this knowledge gap is recommended.

Establishing recommendations for improved farm management and soil conditioning through cotton variety selection is currently not possible. This is because we still know very little about the soil biological environment. Further investigation of the soil biota is warranted to develop tools to predict how soil responds to changes imposed upon it through either crop selection or management. Additionally, over the course of this project we have seen clear evidence that cotton variety choice can have an impact on the soil microbiota. With the yearly release of new varieties the extent and significance of this impact is difficult to gauge. Establishing the extent and nature of these variety differences and their significance for soil microbiota has formed the basis of a new CRDC funded project.

Dr Oliver G G Knox
CSIRO Entomology
Locked Bag 59, Narrabri, NSW 2390
02 6792 1583; oliver.knox@csiro.au

Dr Gupta V S R Vadakattu
CSIRO Entomology
Gate 5, Waite Road, Urrbrae, SA 5064
08 8303 8579; gupta.vadakattu@csiro.au